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Genetics

Ligand-activated site-specific recombination in mice

(inducible gene targeting/Cre recombinase/estrogen receptor/tamoxifen/somatic mutations)

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ABSTRACT **Current mouse gene targeting technology is unable to introduce somatic mutations at a chosen time and/or in a given tissue. We report here that conditional site-specific recombination can be achieved in mice using a new version of the Cre/lox system. The Cre recombinase has been fused to a mutated ligand-binding domain of the human estrogen receptor (ER) resulting in a tamoxifen-dependent Cre recombinase, Cre-ER^T, which is activated by tamoxifen, but not by estradiol. Transgenic mice were generated expressing Cre-ER^T under the control of a cytomegalovirus promoter. We show that excision of a chromosomally integrated gene flanked by *loxP* sites can be induced by administration of tamoxifen to these transgenic mice, whereas no excision could be detected in untreated animals. This conditional site-specific recombination system should allow the analysis of knockout phenotypes that cannot be addressed by conventional gene targeting.**

The study of the genetic control of mammalian development and physiology has been revolutionized by the ability to inactivate (knockout) specific genes by homologous recombination in the mouse (1). However, using current gene targeting technology, interpretations of knockout phenotypes are often limited by several factors. First, the presence of a selection marker may influence the phenotype of the mutation (2, 3). Second, artefacts can arise due to the lack of a gene product for the whole lifetime of the animal. Third, the inactivation of a gene may result in intra-utero lethality, precluding analysis of the possible function(s) of the gene at later stages of development and/or post-natally. A conditional gene targeting method based on the inducible activity of an engineered DNA recombinase could overcome these limitations by allowing the removal of the selection cassette and the timed and tissue-specific inactivation of target genes at will during development and in the adult mouse (4). Furthermore, such an inducible system could help in certain cases to distinguish between anomalies related to a mixed genetic background and those due to mutation of the targeted gene.

The bacteriophage P1 Cre recombinase efficiently excises DNA flanked by two directly repeated *loxP* recognition sites in mammalian cells (5, 6). We have previously reported that fusion of the ligand-binding domain (LBD) of the estrogen receptor (ER) to the Cre recombinase generates a chimeric recombinase whose activity in cultured cells is dependent on the presence of an estrogen (estradiol) or an anti-estrogen (tamoxifen) (7). To achieve conditional gene targeting in mice, where endogenous estradiol is present, we have subsequently fused Cre to a mutated LBD of the human ER (Gly 521 → Arg, G521R) resulting in the chimeric protein Cre-ER^T. Indeed, the corresponding mouse ER LBD mutant (G525R) does not bind 17β-estradiol (E_2), whereas it binds the synthetic ligands tamoxifen and 4-hydroxytamoxifen (OHT) (8). We report here that Cre-ER^T is a functional tamoxifen-dependent recombinase in cultured cells and in transgenic mice.

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MATERIALS AND METHODS

Construction of Plasmids and Generation of Transgenic Mice. pCMVCre-ER^T was constructed by cloning the 2-kb *Eco*RI fragment isolated from pCre-ER^T into the *Eco*RI site of the expression vector pMGSV1. pCre-ER^T was obtained by first replacing the 252-bp *Hin*dIII–*Bgl*II fragment of pCre-ER (7) with the corresponding fragment isolated from the expression vector HEG0 (9), coding for the human ER containing a glycine at amino acid 400. The amino acid corresponding to glycine 521 of the human ER was then mutated to an arginine by site-directed mutagenesis using the oligonucleotide 5′-CACATGAGTAACAAAAGAATGGAGCATCTGTAC-3′. To obtain pMGSV1, a 600-bp *Xba*I–*Hin*dIII restriction fragment containing the enhancer/promoter region of the major IE gene of the human cytomegalovirus (CMV), isolated from pCMVcat (10) was first cloned into BSM+ (Vector Laboratories cloning systems) digested with *Xba*I and *Hin*dIII, resulting in BSM–CMV. The simian virus 40 promoter region of pSG1 (11) was then replaced with the CMV promoter by cloning the 600-bp *Sac*I–*Hin*dIII restriction fragment isolated from BSM–CMV into pSG1 digested with *Sac*I and *Bam*HI, after filling in the *Hin*dIII and *Bam*HI restriction sites with T4 polymerase. The 4.6-kb *Pvu*II DNA fragment of pCMVCre-ER^T was injected into (C57BL/6 × SJL) F_1 zygotes at a concentration of 4 ng/ml to generate transgenic mice according to established procedures (12).

PCR Conditions. PCR amplification was carried out in a buffer containing 10 mM Tris·HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM of each primer, and 2 units *Taq* polymerase using 1 μg of genomic DNA as template. After 35 cycles (30 sec at 94°C, 30 sec at 55°C) the products were analyzed on ethidium bromide-stained 2.5% agarose gels.

Analysis of F9 Cells. Mouse F9 embryonal carcinoma cells carrying a floxed *tkneo* marker integrated into exon 4 of one retinoid X receptor α (RXRα) allele [RXR$\alpha^{+/-(LNL)}$, (13)] were transiently transfected with pCre-ER (7) or pCre-ER^T. After a 24-h incubation period, cells were grown in the presence of vehicle (ethanol) alone, 100 nM E_2, or 1 μM OHT for 48 h. PCR amplification of a 175-bp fragment specific for the excised allele was carried out using 1 μg of genomic DNA as template. The 5′ and 3′ primers were 5′-GGCAAACACTATGG-3′ and 5′-TTGCGTACTGTCCTCTT-3′, respectively.

Genotyping of Mice. The Cre-ER^T transgene and the RXR$\alpha^{\Delta AF2(LNL)}$ target allele were detected in mouse tail DNA

Abbreviations: CMV, cytomegalovirus; Cre-ER^T, fusion protein between the Cre recombinase and a mutated ligand-binding domain of the human estrogen receptor (G521R); ER, estrogen receptor; LBD, ligand-binding domain; OHT, 4-hydroxytamoxifen; RT, reverse transcriptase; RXRα, retinoid X receptor alpha; TKneo, thymidine kinase/neomycin-resistance fusion gene; wt, wild type; HPRT, hypoxanthine phosphoribosyltransferase.

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both by PCR and Southern hybridization. For PCR detection of the Cre-ER^T transgene, the 5′ primer for *cre* was 5′-ATCCGAAAAGAAAACGTTGA-3′ and the 3′ primer was 5′-ATCCAGGTTACGGATATAGT-3′. For Southern blot analysis, tail DNA (10 μg) was digested with *Eco*RI and a 0.7-kb *Bam*HI–*Xho*I *cre* fragment isolated from pCre-ER (7) was used as a probe. For detection of the RXRα$^{\Delta AF2(LNL)}$ allele, the 5′ and 3′ PCR primers for *tkneo* were 5′-GGTTCTCCGGCCGCTTGGGT-3′ and 5′-GAAGGCGATGCGCTGCGAAT-3′, respectively. For Southern blot analysis, tail DNA was digested with *Bam*HI and probed with a 0.7-kb *Sac*I fragment of the RXRα gene.

Detection of Cre-ER^T mRNA Synthesis in Mice. The level of Cre-ER^T mRNA was estimated by reverse transcriptase (RT)-PCR. Total RNA was isolated from mouse tissues by the LiCl/urea method (14). cDNA was synthesized for 20 min at 50°C using 50 units of Moloney murine leukemia virus RT and 1 μg of RNA and was then amplified by 35 cycles of PCR using primers 1 (5′-TTGACCTCCATAGAAGACAC-3′) and 2 (5′-GGCGATCCCTGAACATGTCC-3′), resulting in a 254-bp fragment of the Cre-ER^T cDNA. As an internal control, a 177-bp cDNA fragment of the hypoxanthine phosphoribosyltransferase (HPRT) mRNA was co-amplified in the same reaction using the 5′ and 3′ primers 5′-GTAATGATCAGTCAACGGGGGAC-3′ and 5′-CCAGCAAGCTTGCAACCTTAACCA-3′, respectively.

Detection of Cre-ER^T-Mediated DNA Excision in Mice. PCR and Southern hybridization was used to analyze Cre-ER^T-mediated excision of the floxed *tkneo* marker from the RXRα$^{\Delta AF2(LNL)}$ target allele. PCR primers were primer 3 (5′-CAAGGAGCCTCCTTTCTCTA-3′) and primer 4 (5′-CCTGCTCTACCTGGTGACTT-3′). These primers amplify a 156-bp fragment of the RXRα wild type (wt) allele and a 190-bp fragment of the RXRα$^{\Delta AF2(L)}$ allele. Southern blot analysis was performed using a 2.0-kb *Kpn*I–*Bam*HI fragment or a 1.6-kb *Kpn*I fragment of the RXRα gene as hybridization probes. Genomic DNA was digested with *Bam*HI or *Nco*I.

Quantitation of DNA Excision and mRNA Levels. Semi-quantitative PCR was used to estimate the relative level of Cre-ER^T-mediated gene excision and semi-quantitative RT-PCR was used to estimate Cre-ER^T mRNA expression. For each sample the appropriate cycle number for remaining within the exponential phase was determined by running different cycle numbers (27, 30, 33, and 35). The ratio between the intensity of the bands derived from the excised allele and from the wt allele, as well as the ratio between the intensity of the bands derived from the Cre-ER^T mRNA and from the HPRT mRNA, was determined by laser densitometry of Polaroid photographs of ethidium bromide-stained agarose gels after subtracting the gel background. Only amplification reactions for which these ratios remained constant for at least two consecutive cycle numbers were used for quantitation. The percentage of Cre-ER^T-mediated deletion in a given organ was calculated from the intensities of the bands derived from the RXRα$^{\Delta AF2(L)}$ and wt RXRα alleles using the formula RXRα$^{\Delta AF2(L)}$/RXRα × 100. To estimate relative levels of Cre-ER^T mRNA expression, the Cre-ER^T signal obtained in a given tissue was normalized by division of the signal obtained for the HPRT standard mRNA. The value calculated for the skin was taken as 100%.

RESULTS AND DISCUSSION

To express a tamoxifen-dependent Cre recombinase, we constructed an expression vector, pCre-ER^T, encoding the fusion protein Cre-ER^T consisting of Cre fused to a mutated LBD of the human ER (G521R). The functionality of Cre-ER^T was tested by transient transfection using a mouse F9 embryonal carcinoma "reporter" cell line, which carries a chromosomally integrated "floxed" *tkneo* gene, i.e., a *tkneo* gene that is flanked by two directly repeated *loxP* sites. In these cells, Cre-ER^T excised the *tkneo* gene in the presence of 1 μM of OHT, but not in the presence of 100 nM of E_2 or in the absence of ligand, whereas, as reported (7), Cre-ER excised the target gene both in the presence of E_2 and OHT (Fig. 1). While the present study was in progress, a similar conditional Cre-ER^T recombinase was described (15).

Transgenic mice expressing Cre-ER^T under the control of a CMV promoter were generated (Fig. 2*a*). Out of three transgenic mouse lines, one showed stable maintenance and mRNA expression of the Cre-ER^T transgene in the tail (data not shown) and was used in all subsequent experiments. Mice expressing Cre-ER^T appeared phenotypically normal.

To analyze the efficiency of DNA excision by Cre-ER^T, we crossed Cre-ER^T mice with mice harboring a floxed target gene. This "reporter" line contains one wt allele of the RXRα gene and one modified RXRα allele carrying a floxed *tkneo* selection marker integrated by homologous recombination into the intron located between exon 8 and exon 9 [RXRα$^{\Delta AF2(LNL)}$, Fig. 2*b*, B.M., P. Kastner, and P.C., unpublished work]. After Cre-mediated excision of the marker gene one *loxP* site remains at this locus [RXRα$^{\Delta AF2(L)}$]. The wt RXRα allele and the excised RXRα$^{\Delta AF2(L)}$ allele can be simultaneously detected by polymerase chain reaction (PCR) using one set of primers (Fig. 2*b*). The relative efficiency of excision was estimated by comparing the intensity of the band amplified from the deleted RXRα$^{\Delta AF2(L)}$ allele with that of the band amplified from the wt RXRα allele, which differs in sequence only by the absence of the *loxP* site.

Offspring generated by crossing Cre-ER^T and RXRα reporter mice, which harbored both the Cre-ER^T transgene and the RXRα$^{\Delta AF2(LNL)}$ allele were identified by genotyping of tail biopsies (data not shown). These mice were treated with OHT at the age of 4 weeks and analyzed for Cre-ER^T-mediated DNA excision (Fig. 3*a*). Excision of the floxed marker gene was undetectable in oil-treated control animals (Fig. 3*a Upper* and data not shown), whereas mice injected intraperitoneally (i.p.) with OHT reproducibly showed excision of the floxed target gene in all organs tested except in the thymus (Fig. 3*a Lower* and data not shown). Importantly, in the tail the deleted RXRα$^{\Delta AF2(L)}$ allele was absent before OHT administration to the animal, whereas its presence was detected following OHT treatment [Fig. 3*a Lower*, compare tail(a) with tail]. The excision pattern and the absence of recombination background in control animals was confirmed using different routes of ligand administration (i.p., subcutaneous, orally) and Southern

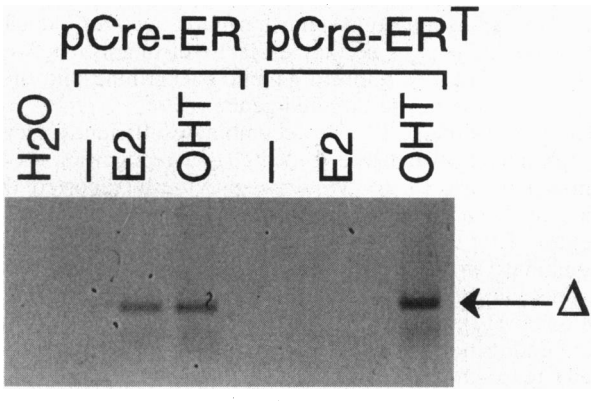

FIG. 1. Ligand-dependent activation of Cre-ER^T in mouse F9 embryonal carcinoma cells. Excision of the *tkneo* gene in RXRα$^{+/-(LNL)}$ cells transfected with a control plasmid encoding an E_2-activated recombinase (pCre-ER) or with a plasmid encoding Cre-ER^T (pCre-ER^T) was analyzed by PCR. Cells were treated with vehicle (−), 100 nM E_2 or 1 μM OHT. A control reaction without DNA template is also shown. The position of the product amplified from the deleted allele (Δ) is indicated.

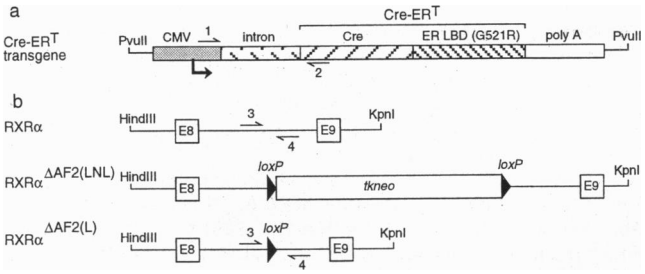

FIG. 2. (*a*) Structure of the Cre-ER^T transgene and strategy for detection of Cre-ER^T mRNA by RT-PCR. The DNA fragment used to generate transgenic mice contained the enhancer/promoter region of the major IE gene of the human CMV promoter, a rabbit β-globin intron, the Cre-ER^T consisting of the Cre recombinase gene fused to the cDNA coding for the G521R mutant of the human ER LBD, and a simian virus 40 polyadenylylation signal [poly(A)]. The positions of the RNA startsite (arrow) and of the primers used for RT-PCR (primers 1 and 2) are indicated. (*b*) Genomic structure of the RXRα wt allele, the RXRα$^{\Delta AF2(LNL)}$ target allele, and the deleted RXRα$^{\Delta AF2(L)}$ allele, and PCR strategy (primers 3 and 4) to analyze Cre-ER^T-mediated excision of the floxed *tkneo* marker. Restriction sites are indicated.

blot analysis (data not shown). These results indicate that Cre-ER^T is a tightly regulated recombinase that displays undetectable activity in the absence of its cognate ligand and can be activated in mice by OHT treatment. We did not observe any deleterious effects of OHT treatment during this study, in agreement with reports indicating that tamoxifen has a very low acute toxicity and causes no severe anomalies in mice (16).

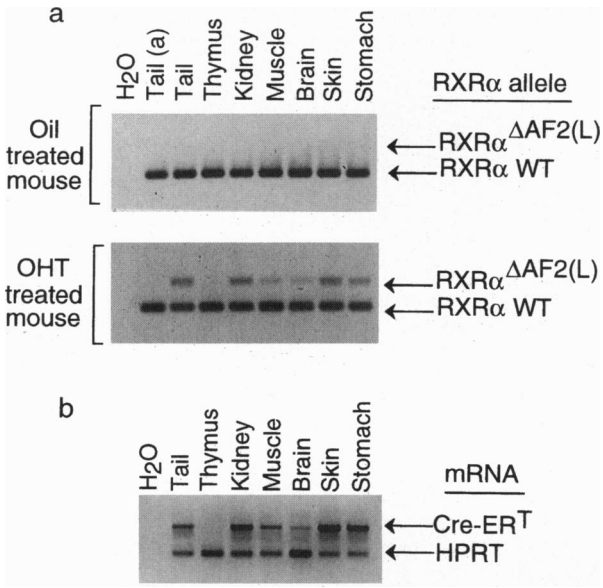

FIG. 3. (*a*) PCR detection of Cre-ER^T-mediated DNA excision in mice. Deletion of the floxed *tkneo* gene in various organs of untreated mice (*Upper*) and OHT-treated mice (*Lower*) was analyzed. PCR was also performed with tail DNA isolated before OHT administration [tail (a)]. Four-week-old Cre-ER^T/RXRα$^{\Delta AF2(LNL)}$ positive littermates were injected i.p. once per day with vehicle (oil) or with 1 mg OHT for 5 consecutive days. One day before the first injection DNA was prepared from tail biopsies of the animals. Two days after the last injection mice were killed and genomic DNA was isolated from various organs. The positions of the PCR products amplified from the RXRα allele and from the deleted RXRα$^{\Delta AF2(L)}$ allele are indicated. (*b*) RT-PCR analysis of Cre-ER^T mRNA expression. RNA was isolated from the same organs of an untreated littermate transgenic for Cre-ER^T, sacrificed on the same day (similar results were obtained when RNA was isolated from OHT-treated animals). PCR products corresponding to the Cre-ER^T mRNA and to the HPRT mRNA used as an internal control are indicated. Reactions run without DNA template are also shown. RT-PCR assays performed without RT did not result in any of the products (data not shown).

Cre-ER^T mRNA was detected in all organs analyzed except in the thymus (Figs. 3*b* and 4), suggesting that the protein is expressed in most tissues. Interestingly, the relative level of Cre-ER^T mRNA correlated well with the level of DNA excision in the various organs examined (Figs. 3 and 4). Excision was most efficient in tail, skin, kidney, and spleen where it occurred for 40–50% of the reporter allele. The level of excision was approximately 30% in the liver and stomach and less than 15% in other organs. In the tail, the excision remained at the same level (≈50%) after three or five injections of OHT (Fig. 4). Assuming that in the tail and possibly also in other tissues Cre-ER^T expression might be restricted to a subset of specific cells, the actual level of excision in these cells could in fact be higher. Note in this respect that CMV-driven transgene expression is known to vary considerably between different cell types in a given organ (17).

Cre-ER^T-mediated gene excision using the present Cre-ER^T expressing transgenic mouse line generates mosaic animals in which cells containing excised and nonexcised target DNA are mixed. Similar mosaics generated with mice homozygous for a targeted gene will allow the analysis of the function of this gene, provided its mutation results in a phenotype that does not depend on the inactivation of the gene in all cells of a given tissue. Furthermore, genetic mosaics can reveal several aspects of the mutant phenotype not necessarily apparent in knockout mutants (18), and also allow the analysis of mutations that result in lethality during embryogenesis or early post-natal development (19). Note that creating Cre-ER^T mouse lines expressing the conditional recombinase under cell/tissue-specific promoters will allow the selective mutation of a given gene at a given time in a given tissue. Note also that using an efficient inducible promoter system [e.g., one based on tetracycline inducibility (20)] that expresses the Cre-ER^T recombinase may be required to ensure that the recombinase levels are high enough to result in 100% excision of the targeted DNA.

Kuehn *et al.* (21) recently reported a method for conditional gene targeting in mice based on regulating the expression of a constitutively active Cre recombinase using an interferon-responsive promoter. The interferon-inducible system resulted in some tissues in higher rates of activated excision than those observed with the present Cre-ER^T mice, but was not as tightly controlled since background recombination was observed in interferon-untreated animals. We believe that further improvement of ligand-dependent Cre recombinase systems sim-

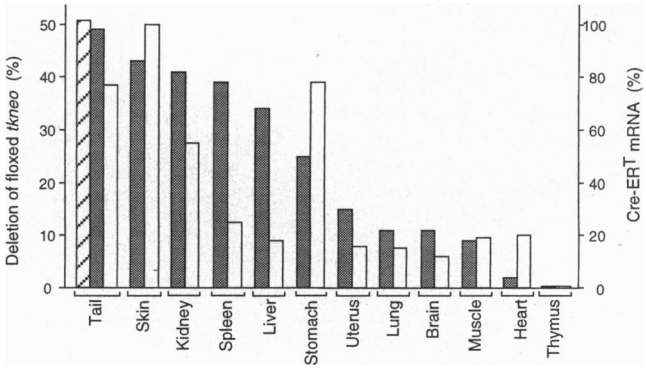

FIG. 4. Pattern of Cre-ER^T-mediated DNA excision and Cre-ER^T mRNA expression in various organs. The level of DNA excision after five i.p. injections of OHT in the indicated organs (shaded bars), the level of DNA excision in the tail 1 day after the third injection (hatched bar), and the corresponding levels of Cre-ER^T mRNA (open bars) are shown. Mice were treated as described in the legend to Fig. 3. No excision could be detected in untreated animals.

ilar to the present one will allow the manipulation of mouse genes to create somatic mutations in a spatio-temporally controlled manner.

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